JOURNAL OF BACTERIOLOGY, June 2000, p. 3254–3258 Vol. 182, No. 11
0021-9193/00/$04.00+0

Characterization of the Streptococcal C5a Peptidase Using a C5a-Green Fluorescent Protein Fusion Protein Substrate

D. K. STAFSLIEN AND P. P. CLEARY*

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota

Received 2 December 1999/Accepted 9 March 2000

A glutathione-*S*-transferase (GST)–C5a–green fluorescent protein (GFP) fusion protein was designed for use as a substrate for the streptococcal C5a peptidase (SCPA). The substrate was immobilized on a glutathione-Sepharose affinity matrix and used to measure wild-type SCPA activity in the range of 0.8 to 800 nM. The results of the assay demonstrated that SCPA is highly heat stable and has optimal activity on the synthetic substrate at or above pH 8.0. SCPA activity was unaffected by 0.1 to 10 mM Ca^{2+}, Mg^{2+}, and Mn^{2+} but was inhibited by the same concentrations of Zn^{2+}. The assay shows high sensitivity to ionic strength; NaCl inhibits SCPA cleavage of GST-C5a-GFP in a dose-dependent manner. Based on previously published computer homology modeling, four substitutions were introduced into the putative active site of SCPA: Asp^{130}-Ala, His^{193}-Ala, Asn^{295}-Ala, and Ser^{512}-Ala. All four mutant proteins had over 1,000-fold less proteolytic activity on C5a in vitro, as determined both by the GFP assay described here and by a polymorphonuclear cell adherence assay. In addition, recombinant SCPA1 and SCPA49, from two distinct lineages of *Streptococcus pyogenes* (group A streptococci), and recombinant SCPB, from *Streptococcus agalactiae* (group B streptococci), were compared in the GFP assay. The three enzymes had similar activities, all cleaving approximately 6 mol of C5a mmol of SCP^{-1} $liter^{-1}$ min^{-1}.

The streptococcal C5a peptidase (SCPA) is a member of the family of subtilisin-like serine proteases, containing a highly conserved Ser-Asp-His catalytic triad, as well as an oxyanion hole asparagine (7, 29). However, SCPA is unlike many other serine proteases in its high degree of substrate specificity (10). Enzymatic studies of SCPA have been significantly hindered by the lack of a simple, reproducible assay. SCPA does not cleave the traditional subtilisin substrates, and its natural substrate, C5a, is costly and difficult to assay. Biological assays for residual C5a activity, such as polymorphonuclear cell (PMN) chemotaxis or adherence, require the tedious isolation of PMNs from whole blood and are difficult to reproduce. In addition, PMNs are often activated by residual lipopolysaccharide or other contaminants present in recombinant SCPA preparations. This background activation can mimic the activation by C5a. Detection of physical changes in C5a is also difficult because SCPA cleaves C5a just seven residues from its C terminus, allowing it to retain its ability to bind most antibodies (10). C5a does not, however, bind PMNs after cleavage by SCPA (20). It is postulated that SCPA contributes to virulence in the early stages of streptococcal disease. Without the C5a chemotactic signal, PMNs are slower to reach the site of infection and the bacteria are better able to colonize the host (17). Since SCPA is an important virulence factor and is expressed on the surface of streptococci, it is the current target of vaccine development for the prevention of both group A and group B streptococcal infections (18).

In this paper, we describe the development of an improved assay for SCPA as well as the use of this assay to characterize the proteolytic activity of wild-type and mutated enzymes. The substrate is a fusion protein consisting of three domains: an "anchor" region (glutathione-*S*-transferase [GST]) for binding to glutathione-Sepharose beads, an SCPA recognition sequence (the entire human C5a peptide), and a reporter (green fluorescent protein [GFP]). The rate of GFP fluorescence released from the solid phase reflects SCPA activity. Four site-directed substitutions (D130A, H193A, N295A, and S512A) were introduced into putative active-site residues of SCPA, and the mutational effects on enzymatic activity were determined.

* Corresponding author. Mailing address: Department of Microbiology, University of Minnesota, Box 196 FUMC, 420 Delaware St., S.E., Minneapolis, MN 55455. Phone: (612) 624-3932. Fax: (612) 626-0623. E-mail: Cleary@lenti.med.umn.edu.

MATERIALS AND METHODS

Strains and culture media. *Escherichia coli* strains DH11S and DH5α were grown in Luria-Bertani broth for cloning and propagation of SCPA and C5a constructs. *E. coli* strain BL21 (Amersham Pharmacia Biotech, Piscataway, N.J.) was grown in 2×YT supplemented with 2% glucose (2×YT-G) for the expression of recombinant fusion proteins (28). All pGEX (Pharmacia) constructs were grown in media that contained 100 μg of ampicillin (Fisher Scientific, Fair Lawn, N.J.) per ml. *Streptococcus pyogenes* strains 90-226 (serotype M1) and CS101 (serotype M49) were grown in Todd-Hewitt broth supplemented with 1% yeast extract (Difco, Detroit, Mich.).

Cloning. The human C5a gene was amplified by PCR from plasmid pBT2006 (a gift from Linda McCarter, University of Iowa) using primers hC5aBamHIfor (5′-CCC CCC GGA TTC AAA GAC GCG CAG ACT AA-3′) and hC5aEcoRIrev2 (5′-CCC CCC GAA TTC CCT TCC CAA TTG CAT GTC TTT ATG AGA GAT-3′). The PCR product was digested with *Bam*HI and *Eco*RI and then ligated into the multiple cloning site of pGEX-4T-1 (Pharmacia). One of the clones, pGhC5a6, was verified by sequencing and chosen for use in further experiments.

The GFP gene was obtained from plasmid pGFPuv (Clontech, Palo Alto, Calif.). The sequence was amplified by PCR using primers gfpSmaIfor (5′-AAA AAA CCC GGG GAG TAA AGG AGA AGA ACT T-3′) and gfpNotIrev (5′-CCC CCC GCG GCC GCT TAT TTG TAG AGC-3′). The PCR product was digested sequentially with *Sma*I and *Not*I and then ligated just downstream of the C5a gene in pGhC5a6. Preparations of plasmid DNA from transformants were screened by restriction digestion with *Bam*HI. Three clones contained the 0.8-kb fragment present only in the recombinant. On plate cultures, these three clones fluoresced green under UV light. One clone, pDSG3, was chosen for use in further studies.

C5a peptidase genes *scpA1* and *scpA49* were amplified by PCR from the chromosomal DNAs of M1 strain 90-226 and M49 strain CS101, respectively. Primers scpfor940 (5′-CCC CCC GGA TCC AAT ACT GTG ACA GAA GAC ACT CC-3′) and scprev4263 (5′-CCC CCC CTC GAG ATG TAA ACG ATT TGT ATC CTT GTC ATT AG-3′) contained *Bam*HI and *Xho*I recognition sites, respectively, to facilitate cloning into the expression vector, pGEX-4T-1. Cloning of recombinant SCPB was done as described previously (9).

Site-directed mutagenesis of *scpA1*. A Transformer (Clontech) site-directed mutagenesis kit was used to introduce four mutations into the cloned *scpA1*

TABLE 1. Mutagenic primers and amino acid substitutions

Primer	Sequence	Amino acid substitution
scpD130A	5′-GAT TTT TAT CAA AAC CAG CAG CAA TCA CTG CAA CAA CAG TC-3′	D130A
scpmutrev1412	5′-GTG TGC CGG CCT CTT GAT CGA-3′	H193A
scpNmutrev2	5′-AAA GCT ACT ATC AGC ACC AGC TGA-3′	N295A
scpmutrev1884	5′-GGC GCA GAC ATA GCA GTT CCA GAA A-3′	S512A

sequence. A switch primer, pgexUSE4287 (5′-CGT ATT GGG CGC TAG CGT GGT TTT TCT TTT CAC-3′), was used to change a unique *Nar*I site in the recombinant plasmid to a unique *Nhe*I site. The mutagenic primers used and the resulting amino acid substitutions in the translated *scpA1* sequence are listed in Table 1. DNA sequences of the mutations were verified by automated fluorescence sequencing at the University of Minnesota Microchemical Facility.

Purification of SCP enzymes. Overnight cultures of *E. coli* strain BL21 carrying recombinant pGEX::*scp* clones were grown in 2×YT-G containing 100 μg of ampicillin per ml, diluted 1/10, and grown to an optical density at 600 nm of between 1.5 and 2.0. Cultures were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (100 μg/ml), incubated for an additional 4 h, and harvested. Cell pellets were stored at −80°C less than 1 week before GST-SCP was extracted. SCP was purified as described in the pGEX manual, except that the Triton X-100 solubilization step was eliminated. SCP was separated from its GST fusion partner by incubation with thrombin at room temperature for 3 h, concentrated, and washed to remove thrombin using a Centricon-100 ultrafiltration apparatus (Amicon, Beverly, Mass.).

Purification of the GST-C5a-GFP fusion protein. GST-C5a-GFP was purified using a modification of the protocol described by Frangioni and Neel (15). Overnight cultures of BL21(pDSG3) were diluted 1/10 in 2×YT-G and grown at 25 to 30°C to an OD_{600} of 0.7 to 0.9. Cultures were induced with 0.2 mM IPTG and vigorous shaking at 25 to 28°C for 4 h. Induced cells were harvested by centrifugation, washed once with a 1/50 volume of STE (150 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA [pH 8]), and frozen at −80°C until needed. Cells were resuspended in 1/100 the original culture volume of STE with 0.1 mg of lysozyme (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml–10 mM $MgCl_2$–50 μg of DNase I (Sigma, St. Louis, Mo.) per ml–Complete Protease Inhibitor (Boehringer) and chilled on ice for 30 min. The suspension was then sonicated three times for 10 s each time at 15 to 20% power with a microtip probe. Sonication was later replaced by one freeze-thaw cycle (−80°C-37°C). After removal of cell debris by centrifugation, crude extracts of GST-hC5a-GFP were stored in 30- to 40-ml aliquots at −80°C until needed. As needed, aliquots were thawed, and the fusion protein was bound to a 1-ml bed volume of glutathione-Sepharose and washed three times with 20 bed volumes of phosphate-buffered saline (PBS). The fusion protein could be stored immobilized on glutathione-Sepharose in 50 mM Tris (pH 7.4)–PBS or PBS with 0.1% bovine serum albumin (BSA) for up to 1 week. GST-C5a-GFP was eluted with 10 mM fresh reduced glutathione in 50 mM Tris (pH 8.0)–0.1% Triton X-100 for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis (see Fig. 1) and for standard curve preparation. GFP fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm on a Tecan Spectrafluor or at an excitation wavelength of 400 nm and an emission wavelength of 508 nm on a Bio-Tek FL600 microplate fluorescence reader.

PMN adherence assay of SCPA activity. The activity of recombinant SCPA, both wild-type and mutant forms, was evaluated using an assay that measures the binding of C5a-activated PMNs to BSA-coated microtiter wells (2). In this adherence assay, wells of a PolySorp plate (Nalge Nunc International, Naperville, Ill.) were coated with 150 μl of 0.5% low-endotoxin BSA (Sigma) in PBS for at least 1 h at 37°C. At the same time, PMNs were isolated from heparinized human blood by layering 5 ml of blood on 3.5-ml Ficoll-Hypaque solution (8% [wt/vol] Ficoll [Sigma], 20% [vol/vol] Hypaque 76 [Nycomed, Princeton, N.J.]). The gradient was established by centrifugation at 300 × *g* for 20 min. PMNs were washed twice in 12 ml of PBS with 2% fetal calf serum. Residual red blood cells were lysed by resuspending the pellet in ice-cold 0.2% NaCl for 30 s and then adding an equal volume of ice-cold 1.6% NaCl. PMNs were centrifuged for 10 min at 300 × *g*, and the pellet was resuspended in 1 ml of ice-cold PBS with 1% glucose. Cells were counted using a hemacytometer (Fisher Scientific).

In a 1.5-ml microcentrifuge tube, 25 pmol of recombinant human C5a (Sigma) and various amounts (13 to 413 fmol) of SCPA were incubated in 0.3 ml of PBS-BSA (0.5% [wt/vol] low-endotoxin BSA) for 45 min at 37°C. At the end of this incubation, the BSA-coated PolySorp plate was washed three times with PBS. The protein mixture (100 μl) and PMNs (100 μl of 2×10^6/ml) were then added to each of the BSA-coated wells in duplicate, and the plates were incubated for 45 min at 37°C. Wells were washed gently with PBS, and adherent PMNs were stained with 50 μl of crystal violet. The cells were lysed with 100 μl of 1% SDS, and the absorbance at 570 nm was read with a Bio-Tek EL340 microplate reader.

SCP kinetic assay. The rate of GFP release by SCP from beads with bound GST-C5a-GFP was calculated over a time period of 25 to 240 min. Reaction mixtures were prepared by adding 20 μl of 50% glutathione-Sepharose with bound substrate as prepared above to 90 μl of PBS–0.1% BSA or 50 mM Tris-HCl (pH 7.4) containing the appropriate amount of SCPA or SCPB. A separate reaction tube was prepared for each time point to ensure constant substrate and enzyme concentrations over the course of the assay. At each time point, the reaction mixtures were mixed and then centrifuged for 5 s to pellet the beads. Seventy-microliter samples were carefully removed from the supernatant and added to 630 μl of PBS in a separate tube to stop the reaction. At the end of the assay, 200-μl aliquots of each sample were added in triplicate to wells of a black microtiter plate for fluorescence detection. GFP fluorescence was then measured.

N-terminal sequencing. Quantities of 400, 200, and 80 pmol of affinity-purified GST-C5a-GFP were incubated with 10 μg of SCPA in 100 μl of 50 mM Tris-HCl (pH 7.4) at 37°C for 12 h. Twenty-microliter aliquots of each reaction mixture were separated on an SDS–12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane for N-terminal sequencing according to the method of Matsudaira (22).

RESULTS AND DISCUSSION

SCPA was cloned, sequenced, and overexpressed in *E. coli* several years ago, but the activity of the enzyme has remained largely uncharacterized due to the lack of a simple, reproducible biochemical assay. Relatively little is known about the enzyme, except that it is highly specific and has a high degree of primary sequence homology to the subtilisin family of serine proteases. SCPA is usually expressed on the surface of group A streptococci; however, in some strains, it is both associated with the cell wall and secreted into the extracellular environment. For example, in some strains, an active fragment of SCPA can be released after exposure to streptococcal cysteine protease (1). The N terminus of this fragment begins at amino acid 90, suggesting that amino acids 32 to 89 are not required for proteolytic activity.

The recombinant SCPA and SCPB enzymes used in this work contain amino acids 32 to 1139, the entire mature protein except for the membrane anchor domain. Enzymes were expressed with N-terminal GST fusion tags for affinity purification and then cleaved with thrombin to remove the GST portion of the molecules. The use of this expression system presumed that SCP does not undergo the N-terminal autocatalytic processing that is characteristic of most subtilases. This assumption was made based on the N-terminal sequence of SCPA purified from streptococci and reported by Chen and Cleary (7). In addition, SCPA purified from the *E. coli* GST expression system showed activity similar to that of the streptococcal enzyme in a PMN adherence assay (data not shown).

To characterize SCPA activity further and to determine the effects of site-directed mutations on catalysis, we developed an assay using a GST-C5a-GFP fusion protein as the substrate. The fusion protein was constructed by cloning the coding regions for human C5a and GFP downstream of GST in pGEX-4T-1. Figure 1 shows GST-C5a-GFP peptide fragments separated by SDS-PAGE after incubation with and without SCPA. A Coomassie blue-stained gel showed that both the 35-kDa GST-C5a and the 28-kDa C5a-GFP cleavage fragments appeared only after incubation with SCPA (Fig. 1A). A Western blot of these same fragments confirmed that both anti-C5a and anti-GST antibodies identified the 63-kDa uncleaved substrate and the 35-kDa cleaved GST-C5a fragment (Fig. 1B and C).

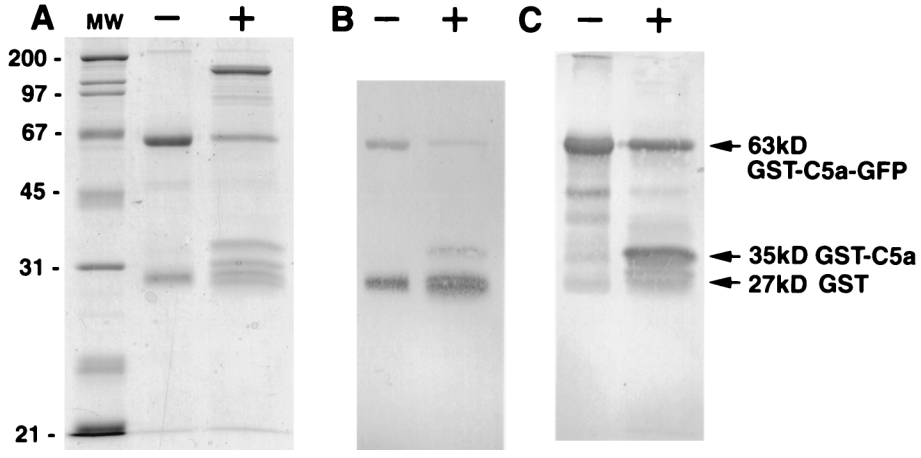

FIG. 1. SDS-PAGE of the GST-C5a-GFP substrate before (−) and after (+) incubation with SCPA. MW, molecular-weight-standard proteins. (A) Coomassie blue-stained gel. (B) Western blot probed with goat anti-GST antibody. (C) Western blot probed with rabbit anti-C5a antibody. The 27-kDa fragment is free GST, which is copurified with GST-C5a-GFP.

As expected, the smaller, 28-kDa C5a-GFP fragment, with only seven amino acids of C5a at its N terminus, was not recognized by either antibody. The 27-kDa GST fragment that appeared in both the Coomassie blue-stained gel and the anti-GST antibody blot may be the result of abortive translation. At the beginning of the human C5a sequence, two rare isoleucine AUA codons are present that may lead to *E. coli* production of a truncated fusion protein that contains only GST and a few amino acids of C5a. The expected cleavage site of the GST-C5a-GFP molecule is at the carboxy terminus of C5a, between His^{67} and Lys^{68}. The 28-kDa C5a-GFP fragment was subjected to N-terminal sequencing; as expected, the peptide contained the seven carboxy-terminal residues of C5a followed by the three residues encoded by the *Eco*RI and *Sma*I restriction sites of pGEX-4T-1 (KDMQLGREFP). The GST-C5a-GFP substrate was also cleavable while bound to glutathione-Sepharose.

Many enzymatic assays using immobilized substrates have been described previously, but they often require radiometric or complex procedures for detection of the reporter (11, 14, 16, 19, 30, 31). The assay described here has the advantage of a simple protocol and quick measurement of the reporter. While GFP is somewhat less sensitive than an enzymatic reporter, it requires no substrate addition or further incubation time to detect the signal. Although the C5a and GFP domains of the recombinant substrate are quite hydrophobic, the fusion protein can be fairly easily purified from *E. coli* and can be stored frozen at −80°C until needed. Based on earlier work demonstrating the capacity of glutathione-Sepharose to bind fusion proteins of various molecular weights, the substrate will bind to the solid support at an estimated concentration of 300 μmol/ml of bed volume (15). This empirical calculation can be confirmed by SDS-PAGE. An end-point assay showed that at a GST-C5a-GFP concentration of approximately 30 mM, SCPA activity could be detected at as low as 0.8 nM in 45 min. The sensitivity of the assay can be increased by a factor of 10 by lengthening the incubation time to 4 h (data not shown).

The GST-C5a-GFP assay was used to assess pH, temperature, ionic strength, and metal cation effects on SCPA activity. At an enzyme concentration of 8.3 nM, optimal SCPA activity was observed at pH 8.0, although high activity was also obtained in carbonate buffer (pH 9.7). SCPA may be sensitive to acidic conditions, with activity toward GST-C5a-GFP dropping off sharply below pH 7. The pH conditions of the assay may not affect enzyme activity but rather alter substrate conformation. However, if the lower pH does affect enzyme activity, this finding could explain two apparently contradictory observations. SCPA was shown to impede the influx of PMNs and phagocytic mononuclear cells during the first 4 h of infection (17). This finding is inconsistent with the classic pyogenic response to streptococci in later stages of throat infections. The hallmark of strep throat is the appearance of white clumps composed of dead phagocytes. If infection proceeds and streptococci lower the local pH by lactic acid production, SCPA may be inactivated, permitting the formation of a C5a chemotactic gradient and the recruitment of substantial numbers of phagocytes to the oral mucosa and tonsils at a later stage of infection.

The heat stability of SCPA activity was also investigated using the GFP assay. SCPA retained greater than 85% activity after incubation at 65°C for 30 min and still exhibited more than 75% activity after 1 h. SCPA even retained 30% activity after incubation in boiling water for 10 min.

Many subtilases require divalent cations for stability or as cofactors. Previous results from our laboratory have shown, however, that EDTA does not significantly affect the activity of SCPA, suggesting that SCPA acts independently of divalent cations (data not shown). To further address this issue, the effect of divalent cations on SCPA activity was measured at an enzyme concentration of 8.3 nM. The proteolysis of GST-C5a-GFP was largely unaffected by magnesium chloride and magnesium acetate at concentrations of 0.1 to 10 mM. Only relatively high concentrations (10 mM) of calcium chloride and manganese chloride were inhibitory. Zinc acetate significantly inhibited SCPA activity at all three of the concentrations tested (10, 1, and 0.1 mM). This inhibition may be the result of zinc oxidation of sulfhydryl groups on either the substrate or the enzyme. Since none of the ions activated proteolysis at the concentrations tested, we conclude that SCPA activity is not dependent on the presence of divalent metal cations.

In contrast, SCPA activity toward the fusion protein substrate showed a surprisingly striking dependence on ionic strength. With sodium chloride in PBS (8.1 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM KCl, 137 mM NaCl [pH 7.4]), enzymatic activity was approximately 50% that observed in 50 mM Tris (pH 7.4). Inhibition of SCP activity increased linearly with sodium chloride concentrations of be-

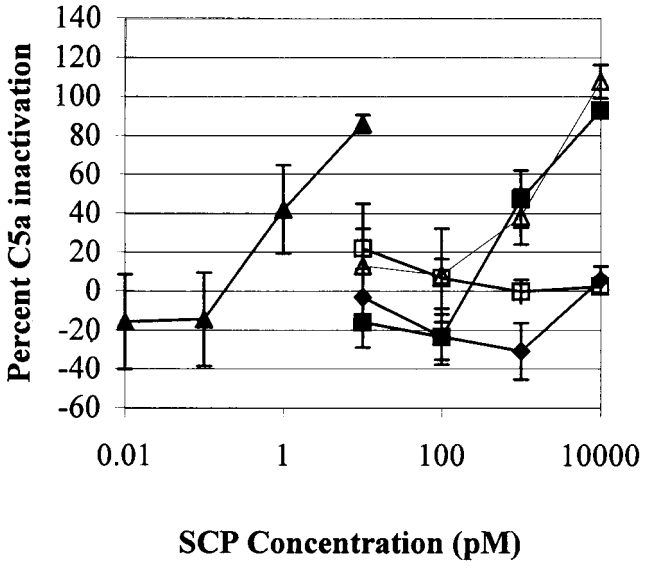

FIG. 2. PMN adherence assays showing that mutant SCPA1 enzymes do not cleave C5a. SCPA1 (wild type or mutant) (0.01 to 10,000 pM) was preincubated with 83 nM C5a and then added to purified PMNs in a 96-well microtiter plate. The adherence of PMNs to BSA-coated microtiter wells in response to residual C5a was measured by staining with crystal violet and measuring the optical density at 570 nm. Symbols: ▲, wild-type SCPA1; ■, D130A; ◆, H193A; △, N295A; □, S512A. Values are the averages from three experiments, and error bars are the standard errors of the mean.

tween 20 and 200 mM in this buffer. Again, it is unclear whether ionic strength affects SCPA itself or merely the folding of the GST-C5a-GFP substrate.

The fluorescent fusion protein was used not only to characterize wild-type SCPA activity but also to compare wild-type and mutant enzymatic activities. Site-directed mutations were introduced into SCPA based on sequence comparisons and computer modeling. Four key residues of SCPA, Ser^{512}, Asp^{130}, His^{193}, and Asn^{295}, were identified and predicted to compose the charge transfer structure of the enzyme (7, 29). Both biochemical and genetic studies have defined the role of these residues in enzymatic activity (3–6, 8, 12, 13, 21, 23–27). Single amino acid substitutions at any of these sites in SCPA resulted in a significant loss of enzymatic activity. When the activities of mutant enzymes (0.1 mg/ml) were compared with that of the wild-type enzyme (1 to 10 ng/ml) in a 4-h kinetic assay, all four mutant enzymes showed a three-log decrease in specific activity as a result of the site-directed substitutions, consistent with reports on similar substitutions in other subtilases. The specific activities of wild-type SCPA1 and of D130A, H193A, N295A, and S512A mutant enzymes were 95 ± 34, 0.031 ± 0.005, 0.055 ± 0.010, 0.057 ± 0.008, and 0.025 ± 0.005 mol of C5a mmol of $SCPA^{-1}$ $liter^{-1}$ min^{-1}, respectively (average of three trials ± standard error of the mean).

Cleavage of SCPA is predicted to eliminate the capacity of C5a to activate PMNs, whereas incubation of C5a with mutant enzymes should not alter chemotactic activity. This notion was confirmed using a traditional PMN adherence assay. The data shown in Fig. 2 demonstrate that the mutant SCPA enzymes were also far less active against recombinant C5a. A 10^3-fold difference between wild-type and mutant enzyme activities was detected for the D130A and N295A mutant enzymes. The S512A and H193A mutant enzymes showed no detectable activity, even at concentrations 10^5-fold higher than that of wild-type SCPA1. The lower sensitivity limit of the C5a-GFP assay prevents the detection of an activity difference this great, but the basic conclusion that each point mutation eliminates at least 99.9% of the proteolytic activity remains the same. While this loss of activity is presumed to be the result of substitutions of active-site residues, the effect of these mutations on protein folding is unknown. Alanine substitutions are not expected to introduce major changes in secondary structure, but the possibility that the native conformation of the enzyme is disrupted by the mutations cannot be eliminated.

A final question that was answered by the fluorometric assay was whether differences between recombinant SCPA1, SCPA49, and SCPB activities from serotype M1 or M49 group A and from group B streptococci, respectively, could be detected. While the sequence homology between SCPA and SCPB is greater than 97%, significantly lower enzymatic activity is associated with group B streptococci than with group A streptococci (9). The enzymes were compared to determine whether this difference is due to intrinsic differences in specific activity that could be due to the few differences in amino acid sequences. The specific activities of recombinant SCPA and SCPB were measured in a kinetic assay. The results showed that the specific activities of the three SCP enzymes were comparable: 6.6 ± 0.7, 6.2 ± 1.1, and 5.8 ± 0.5 mol of C5a mmol of enzyme (SCPA1, SCPA49, and SCPB)$^{-1}$ $liter^{-1}$ min^{-1}, respectively (average of two trials ± standard error of the mean. The difference in the absolute values of the specific activities of SCPA1 in an earlier experiment (see above) and in this experiment is due to batch-to-batch variations in the fluorescence of the reporter and the fraction of cleavable substrate bound to the beads. The data show that in the absence of the signal peptides and membrane anchor domains, the 3% difference in amino acid sequence between SCPA and SCPB does not affect enzymatic activity. Differences in C5a peptidase activity between group A streptococci and group B streptococci must rather be due to differences in surface protein (including C5a peptidase) expression.

SCPA is unusually large relative to subtilisins and many other serine proteases. The reactive center is defined by the N-terminal half of the protein. The carboxy end contains the cell wall and membrane anchor domains (7). Relative to those of other serine proteases, the substrate specificity of SCPA is very limited. We presume that residues located between the active center and the cell wall anchor prescribe the high degree of substrate specificity. This specificity is difficult to explain in terms of molecular evolution. While it seems likely that SCPA must have other substrates besides C5a, one has not yet been found. Over half the mature protein is carboxy terminal to the active-site amino acids, consistent with the possibility that this portion of the enzyme may have other functions. The role of SCPA as an adhesin or invasin is currently under investigation.

ACKNOWLEDGMENTS

We thank Linda McCarter for providing plasmid pBT2006 carrying the human C5a gene.

This work was supported by NIAID grant AI20016 and Wyeth-Lederle Pediatric Vaccines Inc.

REFERENCES

1. **Berge, A., and L. Björck.** 1995. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. J. Biol. Chem. **270:**9862–9867.
2. **Booth, S. A., C. E. Moody, M. V. Dahl, M. J. Herron, and R. D. Nelson.** 1992. Dapsone suppresses integrin-mediated neutrophil adherence function. J. Investig. Dermatol. **98:**135–140.
3. **Brenner, C., A. Bevan, and R. S. Fuller.** 1993. One-step site-directed mutagenesis of the Kex2 protease oxyanion hole. Curr. Biol. **3:**498–506.
4. **Bryan, P., M. W. Pantoliano, S. G. Quill, H. Y. Hsiao, and T. Poulos.** 1986. Site-directed mutagenesis and the role of the oxyanion hole in subtilisin.

Proc. Natl. Acad. Sci. USA **83:**3743–3745.
5. **Carter, P., and J. A. Wells.** 1988. Dissecting the catalytic triad of a serine protease. Nature **332:**564–568.
6. **Carter, P., and J. A. Wells.** 1990. Functional interaction among catalytic residues in subtilisin BPN′. Proteins **7:**335–342.
7. **Chen, C., and P. Cleary.** 1990. Complete nucleotide sequence of the streptococcal C5a peptidase gene of *S. pyogenes*. J. Biol. Chem. **265:**3161–3167.
8. **Chiba, Y., T. Midorikawa, and E. Ichishima.** 1995. Cloning and expression of the carboxypeptidase gene from *Aspergillus saitoi* and determination of the catalytic residues by site-directed mutagenesis. Biochem. J. **308:**405–409.
9. **Chmouryguina, I., A. Suvorov, P. Ferrieri, and P. P. Cleary.** 1996. Conservation of the C5a peptidase genes in group A and B streptococci. Infect. Immun. **64:**2387–2390.
10. **Cleary, P. P., U. Prahbu, J. B. Dale, D. E. Wexler, and J. Handley.** 1992. Streptococcal C5a peptidase is a highly specific endopeptidase. Infect. Immun. **60:**5219–5223.
11. **Craig, D. B., J. C. Wong, R. Polakowski, and N. J. Dovichi.** 1998. General protease assay method coupling solid-phase substrate extraction and capillary electrophoresis. Anal. Chem. **70:**3824–3827.
12. **Craik, C. S., S. Roczniak, C. Largman, and W. J. Rutter.** 1987. The catalytic role of the active site aspartic acid in serine proteases. Science **237:**909–913.
13. **Creemers, J. W., R. J. Siezen, A. J. Roebroek, T. A. Ayoubi, D. Huylebroeck, and W. J. Van de Ven.** 1993. Modulation of furin-mediated proprotein processing activity by site-directed mutagenesis. J. Biol. Chem. **268:**21826–21834.
14. **Fournot, S., F. Roquet, S. L. Salhi, R. Seyer, V. Valverde, J. M. Masson, P. Jouin, B. Pau, M. Nicolas, and V. Hanin.** 1997. Development and standardization of an immuno-quantified solid phase assay for HIV-1 aspartyl protease activity and its application to the evaluation of inhibitors. Anal. Chem. **69:**1746–1752.
15. **Frangioni, J. V., and B. G. Neel.** 1993. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal. Biochem. **210:**179–187.
16. **Jean, F., A. Basak, M. Chretien, and C. Lazure.** 1991. Detection of endopeptidase activity and analysis of cleavage specificity using a radiometric solid-phase enzymatic assay. Anal. Biochem. **194:**399–406.
17. **Ji, Y., L. McLansborough, A. Kondagunta, and P. Cleary.** 1996. C5a peptidase alters clearance and trafficking of group A streptococci by infected mice. Infect. Immun. **64:**503–510.
18. **Ji, Y., B. Carlson, A. Kondagunta, and P. P. Cleary.** 1997. Intranasal immunization with C5a peptidase prevents nasopharyngeal colonization of mice by the group A *Streptococcus*. Infect. Immun. **65:**2080–2087.
19. **Kasten, M., H. Burkhardt, H. J. von Roden, and S. Rauls.** 1989. A spectroscopic collagenase assay using peroxidase-labeled collagen. Anal. Biochem. **176:**150–156.
20. **Kawai, M., D. A. Quincy, B. Lane, K. W. Mollison, J. R. Luly, and J. W. Carter.** 1991. Identification and synthesis of a receptor binding site of human anaphylatoxin C5a. J. Med. Chem. **34:**2068–2071.
21. **Lijnen, H. R., B. Van Hoef, F. De Cock, and D. Collen.** 1990. Effect of fibrin-like stimulators on the activation of plasminogen by tissue-type plasminogen activator (t-PA)—studies with active site mutagenized plasminogen and plasmin resistant t-PA. Thromb. Haemost. **64:**61–68.
22. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
23. **Norioka, S., S. Ohta, T. Ohara, S. I. Lim, and F. Sakiyama.** 1994. Identification of three catalytic triad constituents and Asp-225 essential for function of lysine-specific serine protease, Achromobacter protease I. J. Biol. Chem. **269:**17025–17029.
24. **Pleschka, S., H. D. Klenk, and G. Herrler.** 1995. The catalytic triad of the influenza C virus glycoprotein HEF esterase: characterization by site-directed mutagenesis and functional analysis. J. Gen. Virol. **76:**2529–2537.
25. **Rao, S. N., U. C. Singh, P. A. Bash, and P. A. Kollman.** 1987. Free energy perturbation calculations on binding and catalysis after mutating Asn 155 in subtilisin. Nature **328:**551–554.
26. **Redpath, M. B., T. J. Foster, and C. J. Bailey.** 1991. The role of the serine protease active site in the mode of action of epidermolytic toxin of *Staphylococcus aureus*. FEMS Microbiol. Lett. **65:**151–155.
27. **Register, R. B., and J. A. Shafer.** 1996. A facile system for construction of HSV-1 variants: site directed mutation of the UL26 protease gene in HSV-1. J. Virol. Methods **57:**181–193.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
29. **Siezen, R. J., W. M. de Vos, J. A. Leunissen, and B. W. Dijkstra.** 1991. Homology modeling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Eng. **4:**719–737.
30. **Wondrak, E. M., T. D. Copeland, J. M. Louis, and S. Oroszlan.** 1990. A solid phase assay for the protease of human immunodeficiency virus. Anal. Biochem. **188:**82–85.
31. **Yamamoto, H., and L. J. Murphy.** 1994. Generation of des-(1-3) insulin-1-like growth factor-I in serum by an acid protease. Endocrinology **135:**2432–2436.